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Transcriptome and proteome analysis of ovaries of arrhenotokous and thelytokous *Venturia canescens*

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Abstract

Under arrhenotoky, unfertilized haploid eggs develop as males but under thelytoky they develop into diploid females after they have undergone diploidy restoration. In the parasitoid wasp *Venturia canescens* both reproductive modes occur. Thelytoky is genetically determined but the underlying genetics of diploidy restoration remain unknown. In this study we aim to identify the genes and/or proteins that control thelytoky. cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis of total ovarian RNA and two-dimensional protein electrophoresis in combination with mass spectrometry revealed putative transcripts and proteins involved in arrhenotokous and thelytokous development. The detected tubulin and actin protein differences are most likely functionally related to the two types of reproduction.

Keywords: diploidy restoration, Hymenoptera, messenger RNA, proteins, automictic parthenogenesis.

Introduction

All Hymenoptera have a haplodiploid mode of sex determination. Within haplodiploidy, arrhenotoky is the most common mode of reproduction: unfertilized eggs develop into

males that are haploid, whereas fertilized eggs develop into diploid females. Thelytoky is less common: females develop parthenogenetically from unfertilized eggs after restoration or maintenance of diploidy and males do not occur. Hence, arrhenotokous males and thelytokous females are both produced parthenogenetically under haplodiploidy.

Coexistence of arrhenotokous and thelytokous reproduction raises many evolutionary questions because asexual reproduction has a short-term competitive advantage over sexual reproduction. A thelytokous gene is expected to rapidly spread in a sexual (arrhenotokous) population because it is transmitted twice as efficiently as sexual genes (Maynard Smith, 1978). However, thelytokous populations have the long-term disadvantage of accumulating deleterious mutations (Kondrashov, 1982) and have reduced evolutionary potential to adapt to changing environments (Van Valen, 1973).

Most known cases of thelytoky in Hymenoptera are caused by microorganisms, but a genetic basis for thelytoky has been shown for some species of wasps, ants and the cape honey bee (reviewed in Van Wilgenburg *et al.*, 2006). Lattorff *et al.* (2005) found that in the cape honey bee thelytokous parthenogenesis is a qualitative character determined by a single recessive gene, called *thelytoky* (*th*). Many different forms of thelytoky occur within the Hymenoptera based on cytogenetic mechanisms (Van Wilgenburg *et al.*, 2006), but their molecular mechanisms are completely unknown.

Venturia canescens (Gravenhorst) is a solitary endoparasitoid wasp of lepidopteran larvae. The reproductive mode is a polymorphism that is fixed at the individual level; arrhenotokous females cannot switch to thelytoky or vice versa and females of either mode can occur sympatrically (Schneider *et al.*, 2002). Thelytoky in *V. canescens* is not a result of infection with *Wolbachia* and probably has a genetic basis (Beukeboom & Pijnacker, 2000) but it is not known whether it is determined by a single or multiple genes. To elucidate the genetic basis of thelytoky we compared transcripts and proteins in the ovaries of arrhenotokous and thelytokous females. We used ovaries because the signal for parthenogenetic male or female development appears to be already present in the egg upon oviposition. Diploidy

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Table 1. cDNA-amplified fragment length polymorphism (cDNA-AFLP) differentially expressed fragments in the ovaries of the two reproductive modes with fragment size, GenBank accession number, reproductive mode in which the fragment was found and sequence identities and functions compared to other Hymenoptera

cDNA-AFLP fragment	Size (bp)	GenBank accession no	Reproductive mode	Sequence identities	Function	Species
VcE07	188	AY204698	A	Putative zinc-finger protein (<i>FP</i>)	Hormone response	<i>V. canescens</i>
VcA11	170	GD254135	A	Transducin-like enhancer protein 4	Co-repressor	<i>N. vitripennis</i>
VcC04	127	GD254143	A	LD23562p Uncharacterized conserved protein AY122162	Unknown	<i>N. vitripennis</i>
VcG06	158	GD254171	A	Transcription initiation factor IIA large subunit XP_001599853.1	DNA repair, regulation of transcription	<i>N. vitripennis</i>
VcG07	122	GD254172	A	Calreticulin, partial CG9429	Calcium ion binding	<i>N. vitripennis</i>
VcD08	194	DQ649290	T	Microsatellite Vcan 056	—	<i>V. canescens</i>
VcB09	153	GD254140	T	Adenosylhomocysteinase hydrolase CG11654	Coenzyme transport and metabolism	<i>A. mellifera</i>
VcE08	150	GD254159	T	Flocculin	Flocculation	<i>N. vitripennis</i>

A, arrhenotokous; T, thelytokous.

restoration in the thelytokous strains of *V. canescens* is described as a form of central fusion automictic parthenogenesis where females produce haploid eggs meiotically that subsequently undergo diploidy restoration and develop into diploid females (Speicher, 1937; Beukeboom & Pijnacker, 2000). We do not know what triggers thelytokous eggs to become diploid when they are not fertilized, whereas arrhenotokous eggs remain haploid.

In this study, we aim to identify genes that are differentially regulated during oogenesis in arrhenotokous and thelytokous females of *V. canescens*. We compare the gene and protein expression patterns in the ovaries of unmated females of both reproductive modes and we provide the first candidate genes for thelytokous reproduction in *V. canescens*.

Results

Transcriptome analysis

The total number of differentially expressed cDNA-amplified fragment length polymorphism (cDNA-AFLP) fragments between both reproductive modes from a total of 2104 fragments was 190 (9%) with an average of 3.96 different bands per primer combination. Overall more fragments were found in the arrhenotokous samples indicating that more transcripts are expressed in arrhenotokous than in thelytokous ovaries. Of the differentially expressed fragments 116 were arrhenotokous and 74 thelytokous. Forty-seven arrhenotokous and 49 thelytokous differentially expressed fragments were re-amplified. Subsequently, 92 fragments with good amplification products were sequenced of which 56 (61%) yielded reliable data, but 26 small fragments (<120 bp) were excluded from further analysis as they might represent artefacts.

Thirty sequences (17 arrhenotokous and 13 thelytokous, Supporting Information Appendix SI) were submitted to the NCBI site (<http://blast.ncbi.nlm.nih.gov/Blast>) to search for homologies. A direct nucleotide search revealed two *V.*

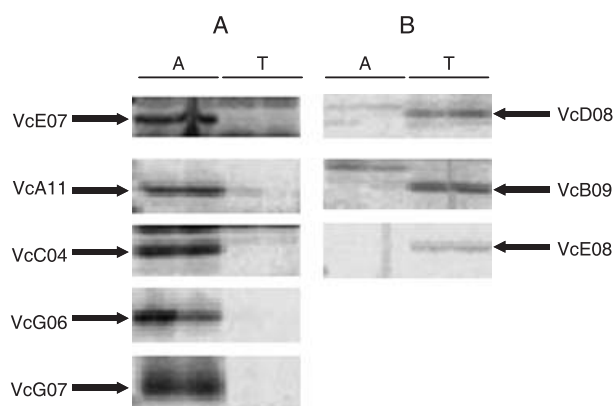


Figure 1. Enlarged view of polyacrylamide gels with differentially expressed cDNA-amplified fragment length polymorphism fragments (cDNA-AFLP). (A) Fragments present only in the ovarian tissue of arrhenotokous females. (B) Fragments present only in thelytokous females. The top row indicates the reproductive mode (A, arrhenotokous; T, thelytokous) loaded into each lane.

canescens matches: fragment VcD08 to a microsatellite flanking region and fragment VcE07 to a zinc finger protein (Table 1). The other sequenced fragments were identified using the NCBI program BLASTX against the protein data-bases of *Apis mellifera* and *Nasonia vitripennis*; the only hymenopterans whose genomes have been sequenced. This search produced six significant hits (Table 1 and Fig. 1).

Proteome analysis

Many ovarian proteins were visualized by two-dimensional gel electrophoresis at pH range 4–7 and differentially expressed spots between arrhenotokous and thelytokous ovaries could be easily identified (Fig. 2). A pilot study showed that this pH range was more informative than pH 3–6. Twenty-one (84%) of the differentially expressed protein spots were only present in the arrhenotokous sample and four only in the thelytokous, in agreement with the observed RNA expression analysis.

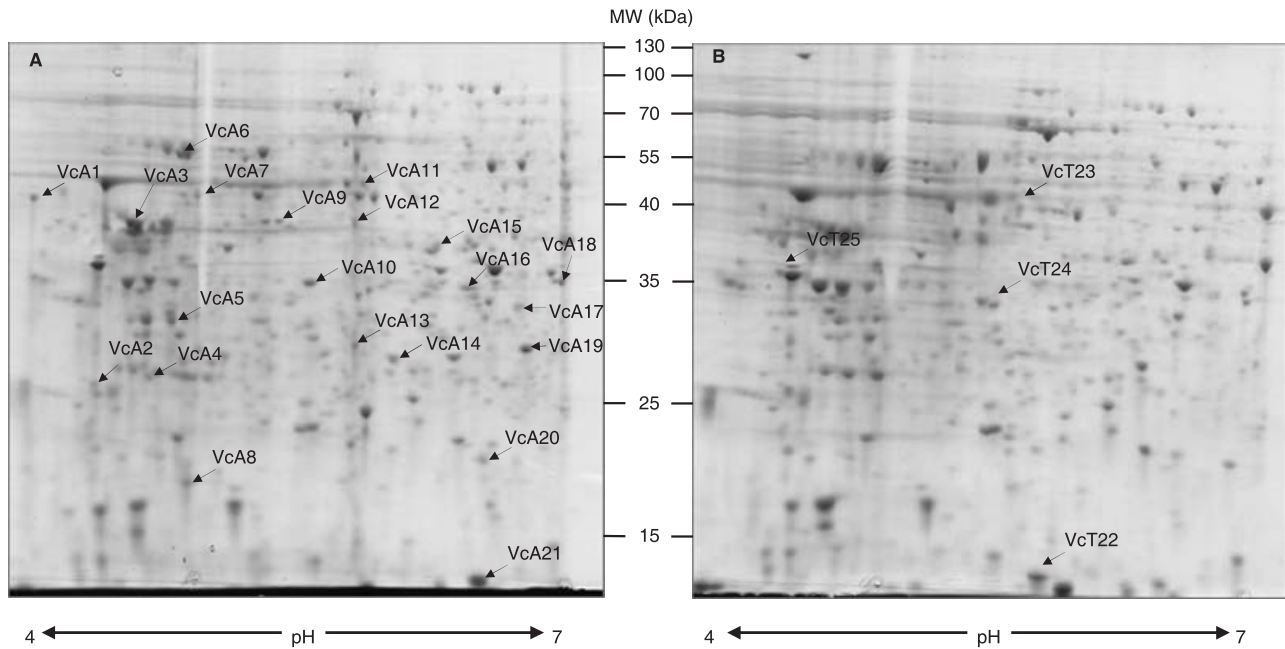


Figure 2. Comparative proteome analyses of arrhenotokous (A) and thelytokous (B) ovaries. Arrows indicate 25 proteins differentially expressed between the two reproductive modes that were separated on pH 4–7 IPG strips and identified by mass spectrometry. A molecular weight (MW) marker with fragment sizes in kilodaltons (kDa) is indicated in the middle of the figure. Details are listed in Table 2.

Table 2. Differentially expressed protein spots in the ovaries of the two reproductive modes with GenBank accession number, total ion score, protein identities and functions and species match

Spot	GenBank accession no.	Total ion score	Protein identities	Function	Species match
A.02	XP392313	208	β -Tubulin at 56D CG9277	Structural constituent of the cytoskeleton	<i>A. mellifera</i>
	gil156538985 re	208	β -1 tubulin, partial	Structural constituent of the cytoskeleton	<i>N. vitripennis</i>
A.12	XP392899	58	Hsp60	Chaperon, posttranslational modification, protein turnover	<i>A. mellifera</i>
	gil156541962 re	58	Chaperonine GroEL	Chaperon, posttranslational modification, protein turnover	<i>N. vitripennis</i>
A.13	gil156551934 re	199	Hypothetical protein	Unknown	<i>N. vitripennis</i>
A.18	XP392465	324	Squid CG16901-PC	RNA recognition, RNA binding protein, gamete generation among other	<i>A. mellifera</i>
	gil156554677 re	215	Heterogeneous nuclear ribonucleoprotein	RNA recognition, RNA binding protein	<i>N. vitripennis</i>
A.19	XP623619	275	Actin-87E, 88F, 5C	Structural constituent of the cytoskeleton, cell motility	<i>A. mellifera</i>
	gil156541054 re	275	RE12057p isoform 1 (Actin)	Structural constituent of the cytoskeleton, cell motility	<i>N. vitripennis</i>
A.20	XP001123058	67	Rbp1-like CG1987-PA	Nucleic acid binding, RNA splicing	<i>A. mellifera</i>
	XP397273	67	Atypical protein kinase C CG10261-PA	Oocyte axis determination among other functions	<i>A. mellifera</i>
	gil156552744 re	66	Eukaryotic membrane protein	Unknown	<i>N. vitripennis</i>
A.21	gil156546944 re	172	Ribosomal protein S12	Structural constituent of the ribosome, translation	<i>N. vitripennis</i>
T.32	gil156538110 re	56	Alpha NAC	Protein binding, involved in oogenesis	<i>N. vitripennis</i>

Spot number indicates reproductive mode.

A, arrhenotokous; T, thelytokous.

A search of a local database for *V. canescens* comprising all 14 published *V. canescens* proteins in GenBank and the RNA and protein sequences derived from this study yielded one match: the virus-like particle protein VLP2 (Reineke *et al.*, 2002).

The possible function of eight protein spots was determined by comparison with the *N. vitripennis* and *A. mellifera* protein database from NCBI (Table 2).

No reversal to sexual reproduction of thelytokous females occurred upon antibiotic treatment as shown by the absence

of male offspring during four generations (645 females). In both arrhenotokous and thelytokous *V. canescens* samples neither *Wolbachia*-specific primers (Supporting Information Fig. S1) nor prokaryotic 16S primers yielded amplification products in a diagnostic PCR (data not shown).

Discussion

Using a transcriptomics and proteomics approach we identified several transcripts and proteins that show a presence–absence differential expression in the ovaries of the two reproductive modes of the parasitic wasp *V. canescens*. Expression products were more abundant in the arrhenotokous samples for both mRNA transcripts and proteins. This can be interpreted as arrhenotokous oogenesis requiring more ovarian gene products than thelytokous development. An arrhenotokous egg may require extra transcripts and proteins for processing the sperm nucleus after fertilization. Differences in mRNA and protein content between arrhenotokous and thelytokous females may not be causally related to diploidy restoration in thelytokous eggs but rather be the result of other processes in which these two types of eggs differ, such as the ability to be fertilized.

We have shown that differences in maternal products are present as both mRNA and proteins, but that identified transcripts and proteins do not match within a reproductive mode. It is known that many maternal mRNAs that code for proteins in the oocyte are stored in the cytoplasm of the egg as ribonucleoprotein particles and are expressed and translated later during early embryogenesis until zygotic transcription begins (Lovett & Goldstein, 1977).

We have identified VLP2 as a spot in the arrhenotokous sample, which confirms the reliability of our study. VLPs are particles that protect the parasitoid egg from the immune system of the host (Feddersen *et al.*, 1986). Reineke *et al.* (2002) described the VLP2 amino acid sequence as a RhoGAP (Rho sub-family of GTPases) domain that activates small guanosine triphosphatases that reorganize the actin cytoskeleton (Bourne *et al.*, 1991). Actin was also found to be differentially expressed in our study. In *Saccharomyces cerevisiae* actin cables act as rails for microtubule recruitment at spindle target sites (Gundersen & Bretscher, 2003). Hence, VLP2 and actin are two possible candidates for causing the difference in chromosome segregation in arrhenotokous and thelytokous eggs.

A protein that shows expression in arrhenotokous but not in thelytokous ovaries is tubulin (*Tub56D*). Tubulin is the main component of the microtubules involved in forming the mitotic spindle. In fertilized oocytes, sperm provides the centrosomes which together with the maternal pericentriolar material organize the microtubules to move the oocyte pronucleus towards the cell centre (Morito *et al.*, 2005). In Hymenoptera, male arrhenotokous parthenogens develop from unfertilized eggs that do not receive paternal centro-

somes, but produce them *de novo* from maternal tubulin sequestered in the accessory nuclei (Ferree *et al.*, 2006). This is consistent with our observation of tubulin overexpression in arrhenotokous ovaries and with the hypothesis that more tubulin is needed to process the paternal centrosomes if the egg is fertilized. However, thelytokous eggs do not accumulate tubulin and must have an alternative way to circumvent the lack of paternal centrosomes inheritance. A likely explanation is that unfertilized thelytokous *V. canescens* eggs can assemble the spindle from already existing cytoplasmic centrioles, as has been described for the hymenopterans *Muscidifurax uniraptor* (Riparbelli *et al.*, 1998) and *N. vitripennis* (Tram & Sullivan, 2000).

Recently, genes related to thelytoky have been identified in aphids and honeybees. Cortés *et al.* (2008) identified a tubulin gene in aphids similar to *Tub56D* involved in the cascade leading to sexual phenotypes. Lattorff *et al.* (2007) identified the transcription factor *grainy head* as the most likely candidate for the *thelytoky* gene in *A. mellifera capensis*. Similar to the cape honey bee, *V. canescens* achieves diploidization of thelytokous eggs by central fusion automictic parthenogenesis (Verma & Ruttner, 1983; Beukeboom & Pijnacker, 2000), but aphid eggs undergo apomictic parthenogenesis. Thus, aphids and the honeybee have two different cytological mechanisms of parthenogenesis which are likely to also have different genetic bases. Although the cytological mechanism in *V. canescens* resembles that of *A. mellifera capensis*, the absence of *grainy head* in our differential analysis suggests yet another genetic basis in *Venturia*.

For the other differentially expressed genes and proteins in our study, it is difficult to assess if and how they play a role in the distinction between the reproductive modes. Further study is needed to confirm whether their expression is functionally related to reproductive mode.

The results of Beukeboom & Pijnacker (2000), together with the antibiotic and PCR results presented in this study, make it unlikely that thelytoky is caused by endosymbionts in *V. canescens* (but see Weeks *et al.*, 2003 for potential pitfalls). This is the first study comparing the transcriptome and proteome in arrhenotokous and thelytokous oocyte development. It revealed several genes that are differentially regulated in arrhenotokous and thelytokous ovaries that may be causally related to diploidy restoration, fertilization or other processes that differ in arrhenotokous and thelytokous eggs. Further studies on the function of these candidate genes are needed to resolve this and to elucidate the genetic mechanism of parthenogenetic reproduction in insects.

Experimental procedures

RNA extraction

Two independent samples of 10 virgin females from arrhenotokous strains, with four individuals from Mont Boron-Valbonne and Valence and two from Antibes, were collected. Similarly, samples

were collected from thelytokous strains, with two individuals from Mont Boron, Mont Boron 2003, Valbonne, Valence and one from Antibes and Antibes 2003. Ovaries were pooled per reproductive mode to ensure that any possible difference in expression between the two reproductive modes was not a result of genetic variation between populations. Ovaries were dissected in phosphate-buffered saline (PBS). Tissue was disrupted in 350 µl RLT lysis buffer (QIAGEN, Valencia, CA, USA) and β-mercaptoethanol using a T25 Basic Ultra-Turrax® homogenizator (IKA Labortechnik, Staufen, Germany). The QIAprep Spin Miniprep Kit (QIAGEN) was used for total RNA extraction following the manufacturer's protocol.

cDNA-AFLP® analysis and sequencing

Double-stranded cDNA was synthesized from 20 µg total RNA for each of the four samples and cDNA-AFLP analysis (Vos *et al.*, 1995; Bachem *et al.*, 1996) was further performed according to Vuylsteke *et al.* (2007) using *TaqI* and *MseI* (Reineke *et al.*, 2003). The analysis was performed in duplicate with two independent samples per reproductive mode which included a biological control and excluded any variation because of technical procedures. A selection of differentially expressed fragments was sequenced. Similarity searches were carried out by comparing the cDNA nucleotide sequences against the reference protein database of *A. mellifera* (genome built 4.0) and *N. vitripennis* (genome built 1.1) using the programs BLASTX and tBLASTN from NCBI, FASTA (Pearson *et al.*, 1997) and WU-BLAST2 (www.ei.ac.uk/blast2/). Homologous sequences with an *E*-value lower than 1×10^{-6} were considered significant (Table 1).

Protein extraction

A preliminary Bradford assay was performed to measure total protein content in ovaries of two arrhenotokous and two thelytokous wasps, yielding 0.02 µg/µl per individual and similar for both reproductive modes.

As for the RNA, samples were pooled from the ovaries of 180 females of each reproductive mode. Samples were dissected in PBS and collected in urea sample buffer [9.5 M urea, 2% 3-[(cholamodopropyl)-dimethylammonio]-1-propanesulfonate, 2 mM tributylphosphine and 0.8% carrier ampholites]. No protease inhibitors were used during dissection and sample preparation. Cells were disrupted with glass beads in a Savant Fast Prep F120 (Bio 101, Vista, CA, USA) and supernatant was collected by centrifugation.

Protein electrophoresis

Isoelectric focusing (IEF) was performed according to Rabilloud *et al.* (1994) using ReadyStrip immobilized pH gradient strip pH 4–7 (Bio-Rad, Hercules, CA, USA). IEF was performed at 50 µA/strip, 150 V for 30 min, 300 V for 1 h, 600 V for 1 h and 8000 V until steady state (22 000 Vh) at 20 °C.

The second dimension electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis) followed Görg *et al.* (2000). Equilibrated IPG strips were fixed to a precast 12.5% polyacrylamide gel (Criterion Tris-HCl gel 13.3 × 8.7 cm, Bio-Rad) with a 0.5% agarose gel solution with a PageRuler™ Prestained Protein Ladder (Fermentas, Burlington, Canada) as reference marker. Electrophoresis was performed at room temperature and 100 V for 2.5 h. Gels were fixed and stained with Coomassie Brilliant Blue (Page Blue, Fermentas) at room temperature for 3 h and subsequent overnight destaining. Gels were analysed and

scanned using the PDQUEST 2D ANALYSIS software (Bio-Rad). Differentially expressed spots were selected based on absence/presence and excised for in gel digestion and matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) analysis.

Mass spectrometry analysis

Gel slices were destained with 50% acetonitrile in 25 mM NH₄HCO₃, dried, rehydrated in trypsin solution (Promega V5111; Promega Madison, WI, USA) with an overlay of 25 mM NH₄HCO₃ and incubated overnight at 37 °C. To recover the tryptic peptides, gel slices were eluted three times with 75% acetonitrile and 0.1% aqueous trifluoroacetic acid (TFA) and concentrated under vacuum. Finally, the dry peptides were dissolved in 0.1% TFA in 5% acetonitrile. MS analysis was performed using a MALDI-TOF/TOF 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA).

Protein identification was performed using MASCOT (www.matrixscience.com), local host (version 1.9, Matrix Science, London, UK), searching against the *A. mellifera* and *N. vitripennis* protein sequence databases at the NCBI. Peptide tolerance was set to 50 p.p.m. and MS/MS tolerance to 0.2 Da, allowing for two missed trypsin cleavages and variable modification for oxidation of methionine (Appendix SII). Results with at least two peptides with a MASCOT score > 95% were considered as significant.

Antibiotic treatment and PCR-screening for *Wolbachia* infection

Females from six thelytokous strains were fed a solution of 1% tetracycline and 10% sucrose during four generations. One arrhenotokous strain and curing of mated females of the parasitic wasp *N. vitripennis* infected with *Wolbachia* (LAB II strain) served as controls. In addition, a PCR assay tested for the presence of microorganisms in ovaries of arrhenotokous and thelytokous females using prokaryotic and *Wolbachia*-specific primers. Several organisms were used as controls of the experimental procedures for establishing presence and absence of *Wolbachia* and other prokaryotes (see Supporting Information Fig. S1). Wasps were dissected under sterile conditions in PBS. Specific primers were used to detect the presence of *Wolbachia* (wspF 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and wspR 5'-AAAAAT-TAAACGCTACTCCA-3', named 81F/691R in Zhou *et al.*, 1998) or prokaryotes (16S ribosomal DNA specific primers B8F 5'-AGAGTTTGATCMTGGCTCAG-3' and U1406R 5'-ACGGGCG-GTGTGTRC-3', Lane, 1991). PCR reactions were performed according to a modified protocol of Zhou *et al.* (1998).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PCR amplification of a *Wolbachia* specific DNA fragment. 1, *Lactococcus lactis*; 2, *Nasonia vitripennis* infected with *Wolbachia*; 3, *Ephestia kuehniella* infected with *Wolbachia*; 4, *L. lactis*–*Venturia canescens* DNA mix; 5, 6, 7, arrhenotokous *V. canescens*; 8, 9, 10, thelytokous *V. canescens*; 11, *N. vitripennis* cured of *Wolbachia*; 12, blank; 13, *Escherichia coli*; 14 m blank. M, size marker.

Appendix S1. Sequences of the cDNA-amplified fragment length polymorphism excised bands.

Appendix S1I-A. Mass spectrometry (MS) results for the *Apis mellifera* comparison. For each spot number and reproductive mode (A, arrhenotokous; T, thelytokous), the accession number of the matched *A. mellifera* contig is indicated as well as protein and peptide information extracted from the MS analysis.

Appendix S1I-B. Mass spectrometry (MS) results for the *Nasonia vitripennis* comparison. For each spot number and reproductive mode (A, arrhenotokous; T, thelytokous), the accession number of the matched *N. vitripennis* protein is indicated as well as protein and peptide information extracted from the MS analysis.

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